

Development and Validation of Swine Embryonic Stem Cells: a Review

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Abstract. The establishment of embryonic cell lines from swine should be useful for studies of cell differentiation, developmental gene regulation and the production of transgenics. This paper summarizes the establishment of porcine (*Sus scrofa*) embryonic stem (ES) cell lines from preimplantation blastocysts and their ability to develop into normal chimaeras. ES cells can spontaneously differentiate into cystic embryoid bodies with ectodermal, endodermal, and mesodermal cell types. Further, culture of ES cells to confluence or induction of differentiation with retinoic acid or dimethylsulfoxide results in morphological differentiation into fibroblasts, adipocytes, and epithelial, neuronal, and muscle cells. These ES cells have a normal diploid complement of 38 chromosomes. Scanning electron microscopy of the ES cells reveals a rounded or polygonal, epithelial-like cell with numerous microvilli. The differentiation of these embryonic cell lines into several cell types indicates a pluripotent cell. Furthermore, chimaeric swine have been successfully produced using such ES cells.

Introduction

The ability to isolate embryonic cells directly from preimplantation mouse embryos and to maintain them *in vitro* has provided a powerful research tool. The ability of embryonic cells to differentiate into a wide variety of cell types has lead to their designation as embryonic stem (ES) cells. ES cells have been derived from the inner cell mass of embryos at the blastocyst stage (Evans and Kaufman 1981; Martin 1981; Bradley *et al.* 1984; Doetschman *et al.* 1985). These cells are able to produce all tissues of a new individual (Nagy *et al.* 1993). Once isolated, ES cells may be grown *in vitro* for many generations. This produces an unlimited number of identical cells capable of developing into fully formed adult chimaeras (Evans and Kaufman 1981; Martin 1981; Bradley *et al.* 1984; Wobus *et al.* 1984; Robertson 1987a). The isolation of ES cells has developed from pioneering studies performed with embryonal carcinoma (EC) cell lines (Stevens 1970; Evans and Kaufman 1981; Martin 1981). Embryonal carcinoma cells were originally derived from embryonic cell tumours or teratocarcinomas (Stevens 1970). Rossant and Papaioannou (1984) showed that both EC and ES cells may differentiate *in vitro* into similar cell types. However, the formation of chimaeric embryos exhibiting phenotypically normal development using EC cells is usually low (Papaioannou *et al.* 1979; Rossant and McBurney 1982), whereas ES cells are more efficient at producing chimaeric mice (Bradley *et al.* 1984). This combined evidence demonstrates that ES cells are a better model than EC cells for studying early mammalian development, and differentiation and for producing transgenic individuals.

The ability of ES cells to serve as vectors for the transfer of foreign DNA in the production of transgenic mice (Gossler *et al.* 1986; Robertson *et al.* 1986) is proving to be a significant aspect of biomedical research. ES cell-mediated transgenesis has some distinct advantages over other transgenic methods. First, the efficiency of producing transgenic animals may be significantly increased with this technology (Gossler *et al.* 1986; Rexroad and Pursel 1988). Secondly, these cells can be transformed *in vitro* with foreign DNA (Thomas and Capecchi 1987; Capecchi 1989; Robertson 1991). Genetically transformed ES cells can be selected and individual cell lines (clonal lines) can be derived from a single cell. Once isolated, these individual cell lines can be screened for recombination (homologous or non-homologous) of exogenous DNA into chromosomal DNA. This provides the opportunity to establish the stable incorporation of the desired gene before chimaera production (Gossler *et al.* 1986; Thomas and Capecchi 1987). When DNA-transformed ES cells are incorporated into the gonads and participate in the production of sperm and eggs, the offspring that are produced by such chimaeric individuals will be transgenic (Bradley *et al.* 1984; Robertson 1987a, 1991).

Production of transgenic pigs from DNA-transformed, individually-derived and screened ES cell lines would allow large numbers of genetically altered pigs to be established. These pigs, if derived from a single ES cell line, would have the transgene integrated into a specific location within the genome. In addition, if homologous recombination is utilized, a single copy of the transgene could be inserted into the genome at a specific site. The use of homologous recombination and site-directed

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mutagenesis can provide precise changes in endogenous genes (Thomas and Capecchi 1987). Insertion of single copies of transgenes could circumvent the problems encountered by integration of multiple gene copies as has been seen with many of the GH transgenic pigs (Rexroad and Pursel 1988). This could potentially allow development of stable, genetically altered strains or lines of swine. Additionally, it may be possible to screen for tissue specific expression of the inserted gene before insertion of the embryonic cells into the embryo (Smithies *et al.* 1985). It also may be possible, with selection markers, to increase the frequency of homologous recombination. This would allow replacement of existing genes with genetically-altered genes designed to improve livestock rather than insertion of genes randomly into the host genome.

Establishment of ES cells from a wide variety of species will allow more flexibility in gene regulation studies and developmental biology research. In addition to the mouse, isolation of embryo-derived cell lines has also been reported from preimplantation embryos of the Syrian golden hamster (Doetschman *et al.* 1988; Piedrahita *et al.* 1990c), rabbit (Giles *et al.* 1991, 1993; Neimann and Strelchenko 1994), mink (Sukoyan *et al.* 1992, 1993), pig (Evans *et al.* 1990; Notarianni *et al.* 1990, 1991; Piedrahita *et al.* 1990a, 1990b; Strojek *et al.* 1990; Gerfen *et al.* 1991; Chen and Wu 1993; Onishi and Youngs 1993a, 1993b; Talbot *et al.* 1993a, 1993b; Gerfen and Wheeler 1994), cattle (Strelchenko *et al.* 1991; Stringfellow *et al.* 1991; Saito *et al.* 1992; Simms and First 1993; Cherny and Mercier 1994; Stice *et al.* 1994; Strelchenko and Stice 1994), and sheep (Handyside *et al.* 1987; Flechon *et al.* 1990; Piedrahita *et al.* 1990a; Tsuchiya *et al.* 1994). Swine offer some distinct advantages over other species because they are immunologically and physiologically more similar to humans and thus serve as a better research model (Phillips and Tumbleson 1986). One particularly important application of this technology is the use of swine as biological reactors or factories to produce human proteins necessary for treatment of genetic or other diseases. Transgenic swine have been shown to produce more than 1 g L⁻¹ of a foreign heterologous milk protein (Wall *et al.* 1991). Since swine may potentially produce up to 10 kg of milk per day, and lactation lasts 7 weeks, one sow may produce 1 kg of a human protein such as clotting Factor IX during her lactation (Wall *et al.* 1991). Another important application of transgenic swine is to produce cells, tissues or organs which contain human antigens or proteins for xenografting.

Finally, by utilizing genetically altered ES cells in nuclear transfer strategies, it may be possible to establish large numbers of genetically identical pigs. Identical animals would be a great advantage to researchers interested in the effects of identified genes, to pharmaceutical

companies interested in the production of gene products such as biological hormones, proteins, tissue antigens and antibodies, to agricultural producers for selection of disease resistance, uniformity in production traits and reproductive performance and to basic scientists and developmental biologists for studies regarding gene expression and growth.

Summary of Recent Studies

In this paper we review the establishment of stable embryonic stem cell lines directly from preimplantation porcine (*Sus scrofa*) blastocysts, the differentiation of these ES cells into numerous cell types, and the embryonic development of swine ES cells into viable chimeric pigs (M. B. Wheeler, L. A. Rund, A. M. Davis, G. Bleck, M. L. Roach, V. L. Jarrell, B. R. White and L. Bidner, unpublished data).

Methods

Embryo collection, culture and stem cell isolation. ES cells were isolated from porcine blastocysts and maintained in culture (Wheeler, Rund, Davis, Bleck, Roach, Jarrell, White and Bidner, unpublished data). Embryos were cultured individually on mitomycin C-inactivated mouse embryonic fibroblasts (STO) monolayers with 2 mL of stem cell medium (SCM; Robertson 1987b; Wheeler, Rund, Davis, Bleck, Roach, Jarrell, White and Bidner, unpublished data).

After 7–9 days in culture, the whole embryonic cell colony was plucked off the feeder layer with a fine glass pipette and partially dis-aggregated in a buffered solution of trypsin containing ethylenediamine tetraacetic acid (trypsin-EDTA) (Robertson 1987b). At passages 2–4, the cells were plated onto a dish with no feeder layer with buffalo rat-liver (BRL)-conditioned stem cell medium (CSCM) (Wheeler, Rund, Davis, Bleck, Roach, Jarrell, White and Bidner, unpublished data). ES cells in CSCM have been maintained for up to 44 passages and extensively characterized.

Preparation of porcine ES cells for chimera production. ES cell colonies were plucked with a fine pipette to dislodge them from the underlying cells. The colonies were then transferred to a solution of trypsin-EDTA. The colonies were placed in CSCM to neutralize the trypsin. The ES cells were gently pelleted by centrifugation and then re-suspended in Dulbecco's phosphate-buffered saline (D-PBS; Wheeler, Rund, Davis, Bleck, Roach, Jarrell, White and Bidner, unpublished data).

Production of chimeras. Differentiation of pluripotent ES cells *in vivo* was tested by their ability to participate in the formation of chimeric offspring. Embryos at the morula, blastocyst and expanded blastocyst stage were injected with ES cells by means of a glass injection needle attached to a micromanipulator as previously described

(Favero and Wheeler 1991). After injection, the embryos were immediately transferred to recipient gilts.

Chimaeric embryos were produced using two coat colour markers: Meishan (black hair with black skin pigmentation) ES cells were injected into Duroc (red-brown hair with pink skin pigmentation) embryos. These combinations allowed for easy visual detection of chimaeric animals. Resultant individuals have patches of different skin and hair colour derived from each embryonic cell lineage.

Results

The production of chimaeric pigs from porcine (*Sus scrofa*) ES cells is described. Participation of blastocyst-derived cells in embryonic development as well as in differentiation of these embryonic cell lines into a variety of cell types indicates a pluripotent stem cell. The ES cell lines derived from pig blastocysts have similar morphology to mouse ES cells. Porcine ES cells grow in colonies with diameters that range from 0.08 mm to >1.5 mm. The individual cells making up these colonies are small (8–12 μ m in diameter), round and dark yet translucent. Their nucleus contains several prominent nucleoli and makes up ~80% of the cell volume. Scanning electron micrographs of the ES cells reveal a rounded or polygonal cell with close association of cells to one another; irregular surfaces and microvilli are evident on the outermost cells of the colony (Gerfen and Wheeler 1994). The doubling time of stable pig ES cell lines ranges from 18 h to 48 h. ES cells can spontaneously differentiate into cystic embryoid bodies with ectodermal, endodermal, and mesodermal cell types (Gerfen and Wheeler 1994). Furthermore, culture of ES cells to confluence or induction of differentiation with 1 μ M retinoic acid or 1% dimethylsulfoxide results in the morphological differentiation into fibroblasts, adipocytes, epithelium, neurons and muscle cells. Karyotype analysis of the ES cells revealed a normal diploid complement of 38 chromosomes.

Results have shown a high percentage (96%) of recipient embryos that received ES cells survived micromanipulation. Furthermore, embryo transfer efficiencies (40%) of micromanipulated embryos were similar to those reported for non-micromanipulated porcine embryos. At birth, 72% of offspring produced after micromanipulation and transfer of embryos exhibited coat-colour chimaerism. Coat colour patterns varied with the individual but included single or multiple areas of black hair, dorsal and ventral striping or a combination of these patterns. Furthermore, skin biopsy revealed chimaerism at the level of the individual hair follicles. Performance testing of coat colour chimaeric swine showed growth rates, measured by average daily gain (ADG, in g) of the

chimaeras to be intermediate between the Duroc (embryo donor) and the Meishan (ES cell line donor) age-matched control pigs. The ADG of the chimaeras was significantly different ($P < 0.05$) from both the Duroc and Meishan control pigs. The chimaera growth rate was about 90% that of the Duroc pigs and higher ($P < 0.001$) than that of Meishan pigs (Ellis and Wheeler, unpublished data). Analysis by means of polymerase chain reaction (PCR) of the cholesterol-7 α -hydroxylase (C7 α H) genetic system (Davis and Wheeler 1994) is currently under way to establish the percentage of chimaerism in the individual tissues (Davis, Rund, Bleck and Wheeler, unpublished data). Chimaeric individuals should contain C7 α H breed-specific polymorphisms from both Meishan and Duroc genotypes. Analysis of germ line chimaerism in these pigs has not been completed at this time.

Discussion

The participation of embryo-derived cells in embryonic development as well as differentiation of these embryonic cells into several distinct cell types indicates a pluripotent cell that is of ES cell origin. Numerous methods have been reported for the validation of ES cell lines from mammals. Studies in mice (Martin and Evans 1975; Evans and Kaufman 1981; Bradley *et al.* 1984; Wobus *et al.* 1984; Doetschman *et al.* 1985; Risau *et al.* 1988) and in Syrian Golden Hamsters (Doetschman *et al.* 1988) have used cell morphology, biochemical markers, the ability to differentiate into various cell types and tissue types and participation in embryonic development as criteria for validating the stem cell nature of embryonic cell lines. In swine, there have been several reports of the isolation of embryonic cell lines (Evans *et al.* 1990; Notarianni *et al.* 1990, 1991; Piedrahita *et al.* 1990a, 1990b; Strojek *et al.* 1990; Gerfen *et al.* 1991; Chen and Wu 1993; Gerfen and Wheeler 1994) and inner cell mass cells (Onishi and Youngs 1993a, 1993b; Talbot *et al.* 1993b) from blastocysts. The criteria used for evaluation of undifferentiated ES cell phenotype in these studies have included morphology (Evans *et al.* 1990; Notarianni *et al.* 1990, 1991; Piedrahita *et al.* 1990a, 1990b; Gerfen *et al.* 1991), ability to differentiate *in vitro* into various cell types and tissue types (Evans *et al.* 1990; Notarianni *et al.* 1990; Piedrahita *et al.* 1990a, 1990b; Gerfen *et al.* 1991; Gerfen and Wheeler 1994), limited embryonic cell analysis of biochemical markers via immunocytochemistry (Evans *et al.* 1990; Piedrahita *et al.* 1990a) and enzyme assay (Talbot *et al.* 1993a; Gerfen and Wheeler 1994).

The most appropriate validation for establishing definitive ES cells is the participation of such cells in embryonic development resulting in the production of live offspring. However, none of the reported cell lines have produced

chimaeric offspring after the re-introduction of these cells into pre-implantation porcine embryos. Chen and Wu (1993) reported re-introduction of ES-like cells into blastocysts and the production of live young. However, chimaerism of the offspring was not definitively established. Furthermore, the reported ES-like cells (Evans *et al.* 1990; Notarianni *et al.* 1990, 1991; Piedrahita *et al.* 1990a, 1990b; Strojek *et al.* 1990; Gerfen *et al.* 1991; Chen and Wu 1993; Gerfen and Wheeler 1994) differ morphologically from those isolated, cultured and re-introduced into swine embryos by our group. Recently, we have reported that a high percentage (96%) of recipient embryos receiving ES cells survived micromanipulation and that 40% of these produced live offspring; at birth, 72% of these pigs exhibited observable coat-colour chimaerism (Wheeler, Rund, Davis, Bleck, Roach, Jarrell, White and Bidner, unpublished data). This is, to our knowledge, the first report of the production of validated chimaeric swine from ES cells.

We have used coat colour markers as previously reported in mice (Mintz 1965) to identify chimaeric offspring. Coat colour markers offer a visually observable system for distinguishing chimaerism (McLaren 1976). Chimaeric pigs were produced by means of two coat colour markers: Meishan (black hair with black skin pigmentation) ES cells were injected into Duroc (red-brown hair with pink skin pigmentation) embryos. These coat colour combinations allowed for easy visual detection of chimaeric animals. Resultant individuals have patches of different skin and hair colour derived from each embryonic cell lineage. Microscopic examination has indicated that chimaerism may extend to the individual hair follicles in the mouse (McLaren and Bowman 1969). Examination of skin from chimaeric swine revealed chimaerism at the level of the individual hair follicles. Coat colour patterns varied between individuals. Patterns included single or multiple areas of black hair, dorsal and/or ventral striping or a combination of these patterns (Wheeler, Rund, Davis, Bleck, Roach, Jarrell, White and Bidner, unpublished data). Similar coat colour patterns have been previously reported for chimaeric mice (Mintz 1967).

Conclusions

The establishment of ES cells from swine should be useful for studies of cell differentiation, development, gene regulation and the production of transgenic swine. ES cell-mediated gene transfer has not been used in domestic livestock mainly because of the lack of established, stable ES cell lines. There are two barriers to overcome to produce transgenic pigs with this technology: (1) establishment of undifferentiated ES cell lines; and (2) the successful transformation of the ES cells with the 'foreign' gene(s). In this paper we summarized progress toward overcoming the first of these barriers, that is, the isolation of

undifferentiated porcine ES cell lines and production of the first chimaeric pigs from cultured ES cells.

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References

- Bradley, A., Evans, M., Kaufman, M. H., and Robertson, E. (1984). Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. *Nature (Lond.)* 309, 255-6.
- Capechi, M. R. (1989). Altering the genome by homologous recombination. *Science* 244, 1288-92.
- Chen, L. R., and Wu, M. C. (1993). Establishment of porcine blastocyst-derived embryonic stem cells and production of chimeras by blastocyst injection. In 'Control of Reproduction in the Pig IV'. (Eds G. R. Foxcroft, M. G. Hunter and C. Doberska.) *J. Reprod. Fertil. (Suppl.)* 48, 94. [Poster.]
- Cheray, R. A., and Merel, J. (1994). Evidence for pluripotency of bovine primordial germ cell-derived cell lines maintained in long term culture. *Theriogenology* 41, 175. [Abstr.]
- Davis, A. M., White, B. A., and Wheeler, M. B. (1994). Polymorphisms in the cholesterol 7- α -hydroxylase gene locus in Meishan and Yorkshire swine. *J. Anim. Sci.* 72, 797.
- Doetschman, T. C., Elstetter, H., Katz, M., Schmidt, W., and Kemler, R. (1985). The *in vitro* development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium. *J. Embryol. Exp. Morphol.* 87, 27-45.
- Doetschman, T., Williams, P., and Maeda, N. (1988). Establishment of hamster blastocyst-derived embryonic stem (ES) cells. *Dev. Biol.* 127, 224-7.
- Evans, M. J., and Kaufman, M. H. (1981). Establishment in culture of pluripotent cells from mouse embryos. *Nature (Lond.)* 292, 154-6.
- Evans, M. J., Notarianni, E., Laurie, S., and Moor, R. M. (1990). Derivation and preliminary characterization of pluripotent cell lines from porcine and bovine blastocysts. *Theriogenology* 33, 125-8.
- Favero, L. J., and Wheeler, M. B. (1991). Production of chimeric animals by microinjection. In 'Handbook of Techniques for Reproductive Studies'. (Eds M. B. Wheeler and P. J. Dziuk.) p. 66. (University of Illinois Press: Urbana, IL.)
- Flechon, J. E., Notarianni, E., Gall, C., Laurie, S., Moore, R. M., and Evans, M. J. (1990). Characterization of ovine and porcine embryonic stem cells. *J. Reprod. Fertil. Abstract Series* 6, 25.
- Gerfen, R. W., and Wheeler, M. B. (1994). Isolation of embryonic cell lines from porcine blastocysts. *Anim. Biotechnol.* (In press.)
- Gerfen, R. W., Fajfar-Whetstone, C. J., and Wheeler, M. B. (1991). Isolation of embryonic cell lines from porcine blastocysts.

- Preimplantation Embryo Development'. (Ed. B. Bavister.) p. 16. [Abstr.] (Springer-Verlag: New York.)
- J. R., Foote, R. H., and Mark, W. (1991). Development of embryo stem cell technology for production of chimeric rabbits. *Biol. Reprod. (Suppl. 1)* 44, 57. [Abstr.]
- J. R., Yang, X., Mark, W., and Foote, R. H. (1993). Pluripotency of cultured rabbit inner cell mass cells detected by isozyme analysis and eye pigmentation of fetuses following injection into blastocysts or morulae. *Mol. Reprod. Dev.* 36, 130-8.
- Chandler, A., Doetschman, T., Korn, R., Serfling, E., and Kemler, R. (1986). Transgenesis by means of blastocyst-derived embryonic stem cell lines. *Proc. Natl Acad. Sci. USA* 83, 9065-9.
- Handyside, A., Hooper, M. L., Kaufman, M. H., and Wilmut, I. (1987). Towards the isolation of embryonic stem cell lines from sheep. *Roux's Arch. Dev. Biol.* 196, 185-90.
- Martin, G. R. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc. Natl Acad. Sci. USA* 78, 7634-8.
- Martin, G. R., and Evans, M. J. (1975). Differentiation of clonal lines of teratocarcinoma cells: formation of embryoid bodies *in vitro*. *Proc. Natl Acad. Sci. USA* 72, 1441-5.
- McLaren, A. (1976). Pigment Patterns. In 'Mammalian Chimeras'. (Ed. A. McLaren.) pp. 52-66. (Cambridge University Press: Cambridge.)
- McLaren, A., and Bowman, P. (1969). Mouse chimeras derived from fusion of embryos differing by nine genetic factors. *Nature (Lond.)* 224, 238-40.
- Milatz, B. (1965). Genetic mosaicism in adult mice of quadruparental lineage. *Science* 148, 1232-3.
- Milatz, B. (1967). Gene control of mammalian pigmentary differentiation. I. Clonal origin of melanocytes. *Proc. Natl Acad. Sci. USA* 58, 344-51.
- Nagy, L., Rossant, J., Nagy, R., Abramow-Newerly, W., and Roder, J. C. (1993). Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. *Proc. Natl Acad. Sci. USA* 90, 8424-8.
- Neimann, H., and Strelchenko, N. (1994). Isolation and maintenance of rabbit embryonic stem (ES) cell like cells. *Theriogenology* 41, 265. [Abstr.]
- Notarianni, E., Laurie, S., Moor, R. M., and Evans, M. J. (1990). Maintenance and differentiation in culture of pluripotent embryonic cell lines from pig blastocysts. *J. Reprod. Fertil. (Suppl.)* 41, 51-6.
- Notarianni, E., Galli, Laurie, S., Moor, R. M., and Evans, M. J. (1991). Derivation of pluripotent, embryonic cell lines from the pig and sheep. *J. Reprod. Fertil. (Suppl.)* 43, 255-60.
- Onishi, A., and Youngs, C. R. (1993a). Comparison of immunosurgery with calcium ionophore treatment for isolation of inner cell masses of porcine blastocysts. *Theriogenology* 39, 274. [Abstr.]
- Onishi, A., and Youngs, C. R. (1993b). Isolation of inner cell mass (ICM) derived cell colonies from intact porcine blastocysts. *J. Anim. Sci.* 71 (Suppl. 1), 74. [Abstr.]
- Papaloannou, V. E., Evans, E. P., Gardner, R. L., and Graham, C. F. (1979). Growth and differentiation of an embryonic carcinoma cell line (C145b). *J. Embryol. Exp. Morphol.* 54, 277-95.
- Phillips, R. W., and Tumbleson, M. E. (1986). Models. In 'Swine in Biomedical Research'. Vol. 1. (Ed. M. E. Tumbleson.) pp. 437-40. (Plenum Press: New York.)
- Piedrahita, J. A., Anderson, G. B., and BonDurant, R. H. (1990a). On the isolation of embryonic stem cells: comparative behaviour of murine, porcine, and ovine embryos. *Theriogenology* 34, 879-901.
- Piedrahita, J. A., Anderson, G. B., and BonDurant, R. H. (1990b). Influence of feeder layer type on the efficiency of isolation of porcine embryo-derived cell lines. *Theriogenology* 34, 865-77.
- Piedrahita, J. A., Gulliespie, L., and Maeda, N. (1990c). Establishing an embryonic stem (ES) cell system utilizing hamster embryos. *Biol. Reprod. (Suppl. 1)* 42, 175. [Abstr.]
- Rexroad, C. E., and Pursel, V. G. (1988). Status of gene transfer in domestic animals. *Proc. 11th Int. Congr. Anim. Reprod. Artif. Insemin.* 5, 29-35. (Natl Assn Animal Breeders: Columbia, MO.)
- Risau, W., Sariola, H., Zerwes, H.-G., Sasse, J., Ekblom, P., Kemler, R., and Doetschman, T. (1988). Vasculogenesis and angiogenesis in embryonic-stem-cell-derived embryoid bodies. *Development* 102, 471-8.
- Robertson, E. J. (1987a). Pluripotent stem cell lines as a route into the mouse germ line. *Trends Genet.* 2, 9-13.
- Robertson, E. J. (1987b). Embryo-derived stem cell lines. In 'Teratocarcinomas and Embryonic Stem Cells: a Practical Approach'. (Ed. E. J. Robertson.) pp. 71-122. (IRL Press: Oxford.)
- Robertson, E. J. (1991). Using embryonic stem cells to introduce mutations into the mouse germ line. *Biol. Reprod.* 44, 238-45.
- Robertson, E., Bradley, A., Kuehn, M., and Evans, M. (1986). Germ-line transmission of genes introduced into cultured pluripotent cells by retroviral vector. *Nature (Lond.)* 323, 445-8.
- Rossant, J., and McBurney, M. W. (1982). The developmental potential of a euploid male teratocarcinoma cell line after blastocyst injection. *J. Embryol. Exp. Morphol.* 70, 99-112.
- Rossant, J., and Papaloannou, V. E. (1984). The relationship between embryonic, embryonal carcinoma and embryo-derived stem cells. *Cell Differ.* 15, 155-61.
- Saito, S., Strelchenko, N., and Neimann, H. (1992). Bovine embryonic stem cell-like cells lines cultured over several passages. *Roux's Arch. Dev. Biol.* 201, 134-41.
- Sims, M. L., and First, N. L. (1993). Production of fetuses from totipotent cultured bovine inner cell mass cells. *Theriogenology* 39, 313. [Abstr.]
- Smithies, O., Gregg, R. G., Boggs, S. S., Koralewski, M. A., and Kuchelapatti, R. S. (1985). Insertion of DNA sequences into the human chromosome β globin locus by homologous recombination. *Nature (Lond.)* 317, 230-6.
- Stevens, L. C. (1970). The development of transplantable teratocarcinomas from intratesticular grafts of pre- and postimplantation mouse embryos. *Dev. Biol.* 21, 364-82.
- Stice, S., Strelchenko, N., Betthausen, J., Scott, B., Jurgella, G., Jackson, J., David, V., Keefer, C., and Matthews, L. (1994). Bovine pluripotent embryonic cells contribute to nuclear transfer and chimeric fetuses. *Theriogenology* 41, 304. [Abstr.]
- Strelchenko, N., and Stice, S. (1994). Bovine embryonic pluripotent cell lines derived from morula stage embryos. *Theriogenology* 41, 304. [Abstr.]
- Strelchenko, N., Saito, S., and Neimann, H. (1991). Towards the establishment of bovine embryonic stem cells. *Theriogenology* 35, 274. [Abstr.]
- Stringfellow, D. A., Gray, B. W., Toivio-Kinnucan, M., Galik, P., Riddell, K. P., Brock, K. V., and Kempainen, R. J. (1991). A continuous cell line established from a preimplantation bovine embryo. *Theriogenology* 35, 275. [Abstr.]
- Strajek, R. M., Reed, M. A., Hoover, J. L., and Wagner, T. E. (1990). A method for cultivating morphologically undifferentiated embryonic stem cells from porcine blastocysts. *Theriogenology* 33, 901-13.
- Sukoyan, M. A., Golubitsa, A. N., Zhelezova, A. I., Shilov, S. Y., Votolin, S. Y., Maximovsky, L. P., Andreeva, L. E., McWhir, J., Pack, S. D., Bayborodin, S. I., Kerkis, A. Y., Mizilova, H. I., and Serov, O. L. (1992). Isolation and cultivation of blastocyst-derived stem cell lines from American Mink (*Mustela vison*). *Mol. Reprod. Dev.* 33, 418-31.
- Sukoyan, M. A., Votolin, S. Y., Golubitsa, A. N., Zhelezova, A. I., Zemenova, L. A., and Serov, O. L. (1993). Embryonic stem cells derived from morular, inner cell mass, and blastocysts of

- mink: comparisons of their pluripotencies. *Mol. Reprod. Dev.* 36, 148-58.
- Talbot, N. C., Rexroad, C. E. Jr, Pursel, V. G., and Powell, A. M. (1993a). Alkaline phosphatase staining of pig and sheep epiblast cells in culture. *Mol. Reprod. Dev.* 36, 139-47.
- Talbot, N. C., Powell, A. M., Nel, N. D., Pursel, V. G., and Rexroad, C. E. Jr. (1993b). Culturing the epiblast cells of the pig blastocyst. *In Vitro Cell Dev. Biol.* 29, 543-54.
- Thomas, K. R., and Capecchi, M. R. (1987). Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. *Cell* 51, 503-12.
- Tsuchiya, Y., Raasch, G. A., Brandes, T. L., Mizochita, K., and Youngs, C. R. (1994). Isolation of ICM-derived cell colonies from sheep blastocysts. *Theriogenology* 41, 321. [Abstr.]
- Wall, R. J., Pursel, V. G., Shamay, A., McKnight, C. W., and Henninhausen, L. (1991). High-level heterologous milk protein in the mammary gland of swine. *Proc. Natl Acad. Sci. USA* 88, 1696-700.
- Wobus, A. M., Holzhausen, H., Jakel, P., and Schöberl, A. (1994). Characterization of a pluripotent stem cell line from mouse embryo. *Exp. Cell Res.* 152, 212-19.

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